LytR-CpsA-Psr proteins in Staphylococcus aureus display partial functional redundancy and the deletion of all three severely impairs septum placement and cell separation

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RESEARCH LETTER

LytR-CpsA-Psr proteins in *Staphylococcus aureus* display partial functional redundancy and the deletion of all three severely impairs septum placement and cell separation

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LytR-CpsA-Psr; *Staphylococcus aureus*; cell division; septum; envelope; autolysis.

Abstract

Staphylococcus aureus contains three members of the LytR-CpsA-Psr (LCP) family of membrane proteins: MsrR, SA0908 and SA2103. The characterization of single-, double- and triple-deletion mutants revealed distinct phenotypes for each of the three proteins. MsrR was involved in cell separation and septum formation and influenced β-lactam resistance; SA0908 protected cells from autolysis; and SA2103, although displaying no apparent phenotype by itself, enhanced the properties of *msrR* and *sa0908* mutants when deleted. The deletion of *sa0908* and *sa2103* also further attenuated the virulence of *msrR* mutants in a nematode-killing assay. The severely defective growth phenotype of the triple mutant revealed that LytR-CpsA-Psr proteins are essential for optimal cell division in *S. aureus*. Growth could be rescued to varying degrees by any one of the three proteins, indicating some functional redundancy within members of this protein family. However, differing phenotypic characteristics of all single and double mutants and complemented triple mutants indicated that each protein played a distinct role(s) and contributed differently to phenotypes influencing cell separation, autolysis, cell surface properties and virulence.

Introduction

The staphylococcal cell envelope is of fundamental importance for growth and cell division, interaction with the environment, pathogenesis, antibiotic resistance and immune evasion. The LytR-CpsA-Psr (LCP) family of cell envelope proteins, which is unique to Gram-positive bacteria (Hubscher *et al.*, 2008), consists of membrane-anchored proteins possessing a very short intracellular N-terminal region, a transmembrane helix and a large extracellular fragment carrying the LCP domain. Different bacterial species have been shown to contain between one and 11 LCP proteins (Hubscher *et al.*, 2008). The existence of multiple different LCP proteins in some bacterial species suggests that there must be degrees of functional variability and/or functional redundancy within this protein family.

LCP proteins generally appear to be involved in envelope maintenance, although their function and the role of the LCP domain remain unknown. LytR attenuates the expression of autolysins in *Bacillus subtilis* (Lazarevic *et al.*, 1992) and is essential for normal septum formation in *Streptococcus pneumoniae* (Johnsborg & Havarstein, 2009). LytR/BrpA in *Streptococcus mutans* is required for correct cell division, and plays a role in autolysis and biofilm formation (Chatfield *et al.*, 2005; Wen *et al.*, 2006). ConR in *Anabenea* sp. is involved in vegetative cell septum formation under specific growth conditions (Mella-Herrera *et al.*, 2010).

The *Staphylococcus aureus* genome contains three proteins carrying the LCP domain: MsrR, SA0908 and SA2103 (Hubscher *et al.*, 2008). All three proteins are upregulated upon cell wall damage and therefore belong to the cell wall stress stimulon (Utaida *et al.*, 2003; McAleese *et al.*, 2006; Dengler *et al.*, 2011). Of these three proteins, only MsrR has been studied previously. *msrR* mutants were shown to produce larger cells and more biofilm and to contain less wall teichoic acids than the wild type. They were also more susceptible to β-lactam antibiotics and attenuated in both a nematode-killing assay and a rat experimental endocarditis model (Hubscher *et al.*, 2009). Although it had been indicated previously that MsrR was a transcriptional attenuator

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(Rossi *et al*., 2003), microarray analysis suggested that *msrR* has no direct regulatory activity (Hubscher *et al*., 2008).

Sequence-based phylogeny separates LCP proteins into several different subgroups, which could reflect functional diversification (Hubscher *et al*., 2008). MsrR clusters in the main LCP subfamily (F1), which also contains Psr from enterococci (Rice *et al*., 2001); SA0908 and SA2103, both group in subfamily F2, together with BrpA from *S. mutans* (Wen *et al*., 2006) and LytR from *B. subtilis* (Lazarevic *et al*., 1992; Hubscher *et al*., 2008).

In this study, we analysed the impact of each of the three *S. aureus* LCP proteins on various envelope-related characteristics and determined the extent to which these proteins can complement each other.

Materials and methods

Strains and culture conditions

The strains and plasmids used are listed in Table 1. Strains were grown in Luria–Bertani broth at 37 °C unless stated otherwise. Erythromycin (10 mg L^{-1}), tetracycline (10 mg L^{-1}), chloramphenicol (10 mg L^{-1}) or ampicillin (100 mg L^{-1}) was added when appropriate.

Mutant construction

Markerless *sa0908* and *sa2103* deletions were generated using the pKOR1 counter selection system (Bae & Schneewind, 2006), to obtain RH53 and PS47, respectively. The primers used are shown in Supporting Information, Table S1. The Δ*sa0908*/Δ*msrR* and Δ*sa2103*/Δ*msrR* double mutants, RH72 and PS60, were obtained by phage 85-mediated transduction of Δ*msrR*::*ermB* from strain JH100 into the corresponding single mutants. The Δ*sa2103*/Δ*sa0908* double mutant PS110 was constructed by sequential markerless deletion of the genes. Transduction of Δ*msrR*::*ermB* into PS110 yielded the triple mutant PS111. Correct gene deletion profiles were confirmed by Southern blot and sequencing. The absence of major genomic rearrangements was demonstrated by pulsed-field gel electrophoresis.

Complementation plasmids

The *sa0908* ORF and promoter region was amplified using the primers sa0908-compF and sa0908-compR (Table S1),

Table 1. Strains and plasmids

Strain	Relevant genotype and/or phenotype	Source or reference
S. aureus		
RN4220	Restriction-deficient derivative of NCTC 8325-4	Kreiswirth *et al*. (1983)
MSSA1112	Clinical isolate, *bla*, Mc^s Pen^r	Entenza *et al*. (1997)
JH100	MSSA1112, Δ*msrR*::*ermB*; Mc^s Em^r	Hubscher *et al*. (2009)
RH53	MSSA1112, markerless *sa0908* deletion mutant	This study
PS47	MSSA1112, markerless *sa2103* deletion mutant	This study
RH86	RH53 complemented with pGC2*sa0908*, Cm^r	This study
PS129	PS47 complemented with pGC2*sa2103*, Cm^r	This study
RH72	MSSA1112, *sa0908/msrR* double mutant	This study
PS60	MSSA1112, *sa2103/msrR* double mutant	This study
PS110	MSSA1112, *sa2103/sa0908* double mutant	This study
PS111	MSSA1112, *sa2103/sa0908/msrR* triple mutant	This study
PS142	PS111 complemented with pGC2*msrR*, Cm^r	This study
PS143	PS111 complemented with pGC2*sa0908*, Cm^r	This study
PS144	PS111 complemented with pGC2*sa2103*, Cm^r	This study
E. coli		
DH5α	F^- ϕ80d/acZΔM15 *recA1*	Invitrogen
Plasmids		
pKOR1	*S. aureus-E. coli* shuttle vector, *ori* pAMα1, *ori* ColE1, *E. coli* Am^r, *S. aureus* Cm^r	Bae & Schneewind (2006)
pKOR1*sa0908*	pKOR1 containing 1 kb each of *sa0908* up- and downstream flanking DNA	This study
pKOR1*sa2103*	pKOR1 containing 1 kb each of *sa2103* up- and downstream flanking DNA	This study
pGC2	*E. coli–S. aureus* shuttle plasmid, *ori* ColE1-*ori* pC194 *bla cat; E. coli* Am^r, *S. aureus* Cm^r	Skinner *et al*. (1988)
pGC2*msrR*	pGC2 containing a 1.3 kb fragment comprising the *msrR* ORF and upstream flanking sequence	Hubscher *et al*. (2009)
pGC2*sa0908*	pGC2 containing a 1.9 kb fragment comprising the *sa0908* ORF and upstream flanking sequence	This study
pGC2*sa2103*	pGC2 containing a 2.1 kb fragment comprising the *sa2103* ORF and upstream flanking sequence	This study
$pmsrR_p - luc+$	pGC2 containing the luciferase gene *luc*+ under the control of the promoter of *msrR*	This study
$psa0908_p - luc+$	pGC2 containing the luciferase gene *luc*+ under the control of the promoter of *sa0908*	Dengler *et al*. (2011)
$psa2103_p - luc+$	pGC2 containing the luciferase gene *luc*+ under the control of the promoter of *sa2103*	This study

Mc, methicillin; Pen, penicillin; Am, ampicillin; Tet, tetracycline; Cm, chloramphenicol; r, resistant; s, susceptible.

digested with EcoRI and cloned into plasmid pGC2, to create plasmid pGC2*sa0908*. Primers sa2103-compF and sa2103-compR were used to amplify the *sa2103* gene and promoter region, which was ligated into the SmaI site of pGC2 to create pGC2*sa2103*. Plasmid inserts were confirmed by sequencing.

Northern blot analysis

Total RNA isolation and Northern hybridization were performed as described previously (Hubscher *et al.*, 2009). The primers used for probe amplification are listed in Table S1.

Primer extension

Primer extension reactions were performed as described in (McCallum *et al.*, 2010). Reactions included 20 μg of total RNA from MSSA1112 that was grown to $OD_{600\,nm}$ 1.0 and induced with 1 mg L^{-1} of oxacillin for 30 min and the 5′-biotin-labelled primers sa0908-pe1 and sa2103-pe1 (Table S1). RNA samples used for primer extension were harvested from cultures induced with oxacillin as this is known to induce the cell wall stress stimulon, hence increasing the transcript abundance of *sa0908* and *sa2103* (Dengler *et al.*, 2011).

Luciferase reporter–gene fusion assays

The promoter regions of *msrR*, *sa0908* and *sa2103* were amplified using the primer pairs JR13/JR14 (Rossi *et al.*, 2003), sa0908.lucF/sa0908.lucR (Dengler *et al.*, 2011) and sa2103.lucF/sa2103.lucR (Table S1), respectively. Promoter fragments were digested with Asp718 and NcoI and ligated directly upstream of the promoterless luciferase (*luc*+) gene in vector pSP − *luc*+(Promega). Fragments containing the promoter − *luc*+translational fusions were then excised with Asp718 and EcoR1 and cloned into the *E. coli–S. aureus* shuttle vector pBUS1. The fusion plasmids, p$msrR_p$ − *luc*+, p$sa0908_p$ − *luc*+ and p$sa2103_p$ − *luc*+, were transformed into *S. aureus* RN4220 and reisolated plasmids were further transformed into *S. aureus* MSSA1112.

To determine luciferase activity over growth, three separate culture broths for each mutant were inoculated with overnight cultures to an OD of 0.05 and grown for 9 h. Samples were collected hourly and luciferase activity was measured as described previously (McCallum *et al.*, 2011).

Transmission electron microscopy (TEM)

Bacteria were grown to $OD_{600\,nm}$ 1.0 and processed as described previously (Hubscher *et al.*, 2009).

Induced autolysis

Cells were harvested at $OD_{600\,nm}$ 1.0, washed once with 0.9% NaCl and resuspended in 0.03 M phosphate buffer (pH 6.8) to an $OD_{600\,nm}$ 0.7. Triton X-100 was added to a final concentration of 0.05% to stimulate autolysis (Höltje & Tomasz, 1975; Cornett & Shockman, 1978). The cells were then incubated at 37 °C and 180 r.p.m. and the $OD_{600\,nm}$ was measured over 3 h. Experiments were performed at least in duplicate.

Resistance tests

Qualitative differences in resistance levels were investigated on antibiotic gradient plates (Hubscher *et al.*, 2009). Experiments were performed at least in duplicate.

Self-agglutination

Bacteria were grown in BHI supplemented with 1% glucose to $OD_{600\,nm}$ 4.0. Culture aliquots were then transferred to glass tubes and the $OD_{600\,nm}$ of the top layer was measured in 30-min intervals. Experiments were performed at least in duplicate.

Biofilm

Adhesion to polystyrene dishes was performed as described previously (Hubscher *et al.*, 2009). Experiments were performed at least in duplicate.

Caenorhabditis elegans killing

Caenorhabditis elegans killing assays were performed as described previously (Hubscher *et al.*, 2009).

Bioinformatic tools

The calculation of molecular weight and isoelectric point was performed using the Protean tool from the DNASTAR LASERGENE software (DNASTAR Inc., Madison, WI). For the prediction of transmembrane segments, the TMHMM Server v. 2.0 of the Center for Biological Sequence Analsysis at the Technical University of Denmark at http://www.cbs.dtu.dk/services/TMHMM was used.

Results

Genetic organization of *sa0908* and *sa2103*

SA0908 and SA2103 are highly conserved throughout all published *S. aureus* genomes, exhibiting 95% and 100% amino acid identity between individual strains, respectively. Both *sa0908* and *sa2103* are framed by genes encoding proteins involved in cell envelope functions (Fig. 1). Downstream of *sa0908* lies *sa0905*, encoding the major bifunctional autolysin Atl (Oshida *et al.*, 1995); upstream and

divergently transcribed is *sa0909* (*fmtA*), encoding a low-affinity penicillin-binding protein modulating methicillin resistance and involved in biofilm formation (Fan *et al.*, 2007). Downstream of *sa2103* is *sa2100,* which shares 84% similarity to the amidase domain of autolysin E of *Staphylococcus epidermidis* (Heilmann *et al.*, 1997).

The *sa0908* gene encodes a deduced protein of 405 aa with a predicted molecular weight of 45.7 kDa and a pI of 6.3. The predicted molecular weight of SA2103 is 34.7 kDa and the pI is 9.7. Both are predicted to have a short cytoplasmic tail adjacent to a single transmembrane region, followed by the extracellular part containing the LCP domain, extending from aa 86 to 234 in SA0908 and from aa 90 to 236 in SA2103.

The transcriptional start sites (TSS) of *sa0908* and *sa2103* were identified by primer extension and were 99 and 44 bp upstream of the start codons, respectively, and were preceded by putative promoter elements (Fig. 1a and b). Northern blots revealed that *sa0908* and *sa0907* were co-transcribed on a single mRNA of ~2000 nt in wild-type MSSA1112. The deletion of *sa0908* in strain RH53 resulted in a shorter, ~800 bp, *sa0907* transcript (Fig. 1c). Two transcripts hybridized to the *sa2103* DIG-probe, an ~1100 bp transcript, which initiated at the TSS, and a larger transcript of ~2000 bp, representing a bicistronic *sa2104–sa2103* transcript, which decreased in size to ~1000 bp in the Δ*sa2103* mutant PS47 (Fig. 1d). Promoter–luciferase fusion constructs were used to compare the relative expression levels of *msrR*, *sa0908* and *sa2103* over growth (Fig. 1D). The expression of all three genes peaked during exponential growth when cells were dividing rapidly, and then decreased as cultures entered the stationary phase. The relative expression levels of *msrR* were much higher than those of *sa0908* and *sa2103*.

Growth characteristics

To obtain a comprehensive overview of the functions of LCP genes, we created all possible combinations of double mutants: RH72 (Δ*sa0908*/Δ*msrR*), PS60 (Δ*sa2103*/Δ*msrR*) and PS110 (Δ*sa2103*/Δ*sa0908*), and a triple mutant PS111 (Δ*sa2103*/Δ*sa0908*/Δ*msrR*). To further investigate the roles of individual LCP proteins, we complemented the triple mutant with *msrR, sa0908* or *sa2103* in trans.

The deletion of *msrR* was previously shown to have no effect on the growth rate (Hubscher *et al.*, 2009). The deletion of *sa0908* or *sa2103* also had only a small, but complementable effect on growth in RH53 (Δ*sa0908*) and PS47 (Δ*sa2103*). The deletion of a second LCP protein had negligible further effects on the growth characteristics (data not shown).

The growth of the triple mutant PS111 was severely retarded, with the growth rate decreasing from 1.39 to 0.95 h^{-1} at 37 °C (Fig. 2a). This growth defect was further exacerbated at 42 °C (Fig. 2b). The ability of the three proteins to complement this growth defect differed,

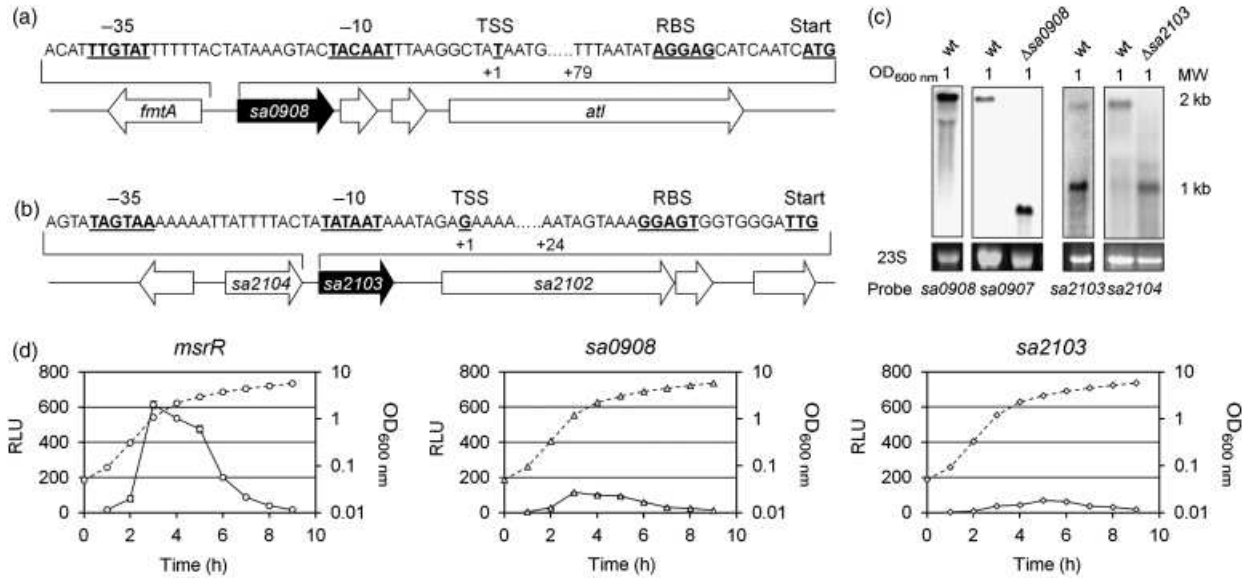

Fig. 1. Genetic organization and transcription of *sa0908* and *sa2103*. Sequence upstream of (a) *sa0908* and (b) *sa2103* shown above maps of the chromosomal regions containing the ORFs. TSS, transcriptional start site; RBS, ribosome-binding site; ATG/TTG, start codons. Numbers indicate the distance from the TSS. (c) Northern blots of *sa0908/sa0907* and *sa2104/sa2103* transcripts. RNA was sampled at $OD_{600\,nm}$ values indicated and 23S rRNA gene bands are shown beneath blots as an indication of RNA loading. (d) Relative expression levels of *msrR*, *sa0908* and *sa2103* over growth. Cultures of MSSA1112 containing $pmsrR_p - luc+$, $psa0908_p - luc+$ and $psa2103_p - luc+$ were grown for 9 h and the $OD_{600\,nm}$ (dashed line) and relative light units (RLU; continuous line) were measured hourly. All values shown represent the mean ± SD obtained from three independent cultures.

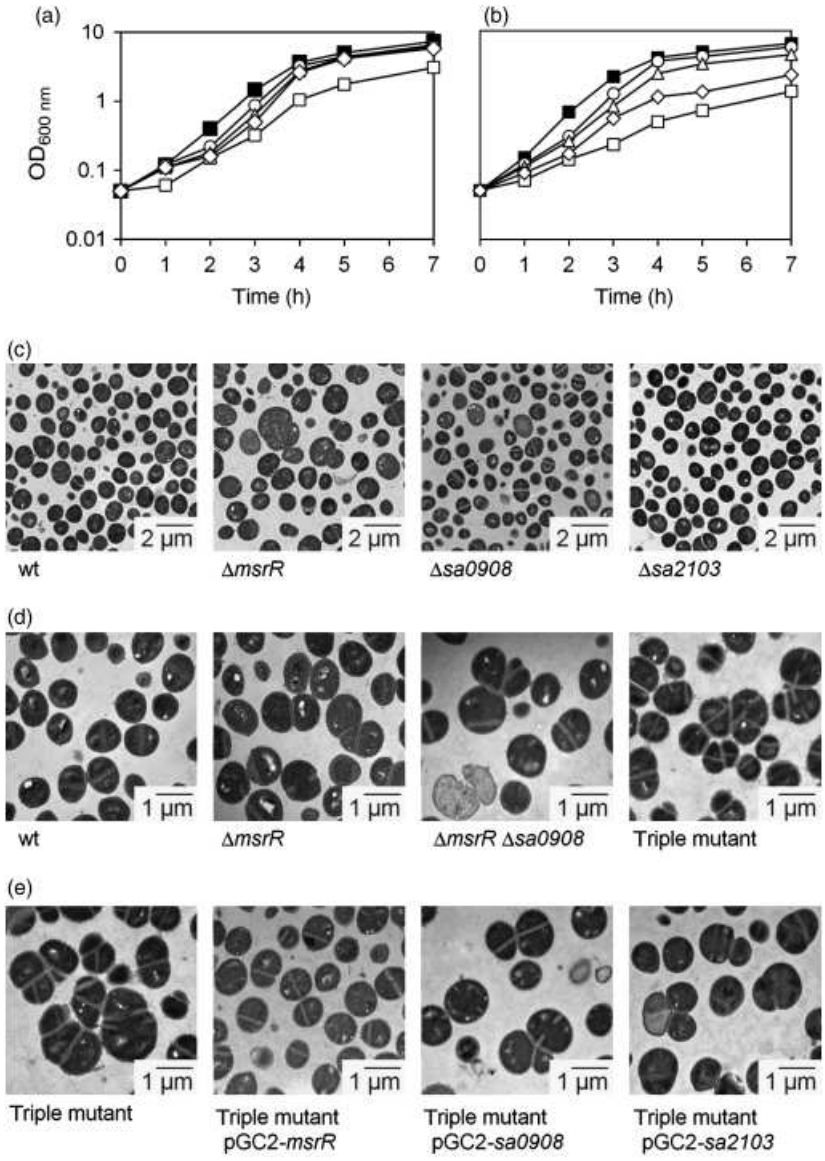

Fig. 2. Growth and TEM. (a) Growth at 37 °C; (b) at 42 °C: full square, wild-type MSSA1112; empty square, triple mutant PS111; empty circle, triple mutant complemented with MsrR (PS142); empty triangle, triple mutant complemented with SA0908 (PS143); empty diamond, triple mutant complemented with SA2103 (PS144). TEM of cells sampled at $OD_{600\,nm}$ 1.0: (c) wild type and single mutants; (d) *msrR,* double and triple mutants; (e) triple mutant complemented with each LCP gene.

especially at the elevated temperature of 42 °C: MsrR restored growth almost to the wild-type level, followed by SA0908, which compensated growth to up to ~70% of the wild type $OD_{600\,nm}$ after 7 h, while SA2103 had the lowest effect (Fig. 2b).

LCP proteins affect cell separation

LCP mutants were analysed by TEM and the cell sizes of a minimum of 100 cells per strain were measured and expressed as the mean ± SD. In single mutants, enlarged cells and irregular septa were observed in the *msrR* mutant (JH100 $\varnothing 1.33 \pm 0.16\,\mu m$) as reported previously (Hubscher *et al.*, 2009). The cells of *sa0908* (RH53 $\varnothing 1.04 \pm 0.07\,\mu m$) and *sa2103* (PS47 $\varnothing 1.04 \pm 0.08\,\mu m$) mutants were indistinguishable from the wild type ($\varnothing 1.05 \pm 0.10\,\mu m$) (Fig. 2c). Cell separation became even more defective in the *sa0908/msrR* double mutant RH72 and was severely aberrant in the triple mutant PS111 (Fig. 2d), which had giant cells with multiple, misplaced septa, precluding accurate cell size measurements.

The PS111 cell separation phenotype could be at least partially complemented by any one of the single wild-type alleles (Fig. 2e). MsrR had the strongest impact, restoring PS142 cells to a wild-type size ($\varnothing 1.09 \pm 0.09\,\mu m$) and septum placement. Complementation with SA0908 (PS143) increased septum regularity and cell separation, but cells were still enlarged ($\varnothing 1.38 \pm 0.19\,\mu m$). Complementation with SA2103 (PS144) had the weakest effect, as although cell separation increased, septal formation

remained quite irregular and individual cell sizes were difficult to measure.

SA0908 protects against autolysis

Growth and cell separation are dependent on the tightly regulated action of autolysins. In single mutants, the deletion of *msrR* or *sa2103* had no effect, while the deletion of *sa0908* increased triton X-100 induced autolysis (Fig. 3a). The deletion of either *msrR* or *sa2103* in *sa0908* mutants further induced autolysis, while the double deletion of *msrR* and *sa2103* had only a marginal impact (Fig. 3b). SA0908 therefore seemed to confer a dominant protective effect against induced autolysis, with MsrR and SA2103 only contributing in minor ways.

The mechanism leading to increased autolysis in the *sa0908* mutant RH53 did not appear to result from altered autolysin activities, because the zymogram profiles of MSSA1112 and RH53 were indistinguishable, regardless of the source of the cell wall extract (MSSA1112 or RH53) used (data not shown). Transcriptional profiles of autolysin genes (*atl*, *fmtA*, *lytM*, *sle1*) and regulators of autolysins such as *sarA* or *graS* in RH53, the only single mutant with altered autolysis, were also very similar to those of the wild-type MSSA1112 by Northern blots (data not shown).

Conversely, the deletion of all three proteins abolished induced autolysis, making PS111 even more resistant to autolysis than the wild type. Complementation with any one of the three LCP genes increased induced autolysis again, with complementation by MsrR resulting in the highest autolysis levels (Fig. 3c).

LCP proteins and β-lactam susceptibility

MsrR deletion is known to reduce oxacillin resistance levels in methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains (Rossi *et al.*, 2003; Hubscher *et al.*, 2009). Because the mutants analysed here are in an MSSA strain background, the resistance phenotypes of all single, double and triple mutants were compared on oxacillin gradient plates to allow the visualization of small differences in growth and resistance. Of the three LCP genes, only *msrR* inactivation increased susceptibility, as seen in the single mutant JH100, in the double mutants RH72 and PS60 and in the triple mutant PS111 (Fig. 3d). The *sa0908/sa2103* double mutant seemed slightly more resistant than the wild type; the reasons for this phenotype are currently unknown, but some compensatory event may have been triggered by the deletion of both genes that influences resistance. The triple mutant PS111 grew very poorly and was hypersusceptible to oxacillin. Complementation of PS111 with any of the three LCP proteins considerably improved growth and increased oxacillin resistance, to different extents: SA2103 < SA0908 < MsrR (Fig 3d).

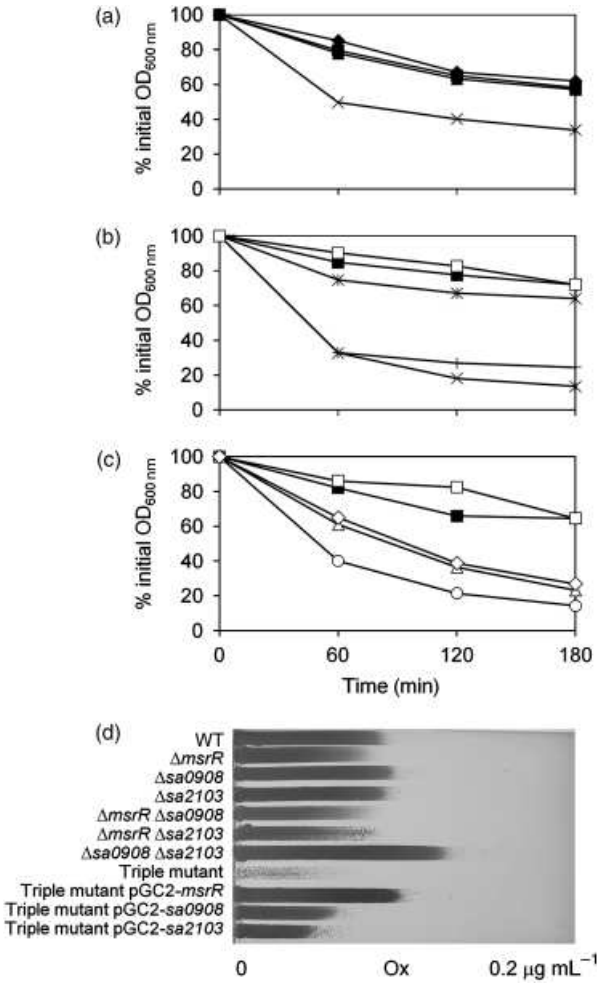

Fig. 3. Triton X-100 induced autolysis and complementation of oxacillin susceptibility. Representative graphs from a minimum of three autolysis experiments comparing the autolytic activities of (a) wild type and single mutants; (b) wild type, double and triple mutants; (c) wild type, triple mutant and complemented triple mutant strains. Full square, MSSA1112; full circle, *msrR* mutant JH100; full triangle, *sa0908* mutant RH53; and full diamond, *sa2103* mutant PS47; cross, *sa0908/msrR* double mutant RH72; star, *sa2103/msrR* double mutant PS60; plus, *sa2103/sa0908* double mutant PS110; empty square, triple mutant PS111; empty circle, triple mutant complemented with MsrR PS142; empty triangle, with SA0908 PS143; or empty diamond, SA2103 PS144. (d) Oxacillin gradient plate (0–0.2 mg L^{-1}) of wild type and all single and double mutants, the triple mutant PS111, and the triple mutant complemented with each single LCP protein.

Agglutination and biofilm formation

MsrR inactivation increases self-agglutination and biofilm formation (Hubscher *et al.*, 2009). Sedimentation due to self-agglutination increased in RH53 (Δ*sa0908*) to

42.6 ± 12%, and in PS47 (Δ*sa2103*) to 18.8 ± 10.8% after 1 h, compared with 8.9 ± 0.7% in wild-type MSSA1112. Sedimentation was further enhanced in double (data not shown) and triple mutants. Complementation of the triple mutant PS111 with each of the three LCP proteins reduced sedimentation with increasing efficiency from SA2103 < SA 908 < MsrR (Fig. 4a).

The *msrR* mutant was the only single mutant to measurably produce more biofilm. In double mutants, biofilm was only enhanced in combination with *msrR* inactivation (data not shown). Almost no biofilm was observed in the triple mutant, but complementation by each single LCP protein restored varying levels of biofilm formation (Fig. 4b and c). The strongest biofilm was produced by SA2103 complementation in strain PS144, followed by complementation with SA0908 and then MsrR.

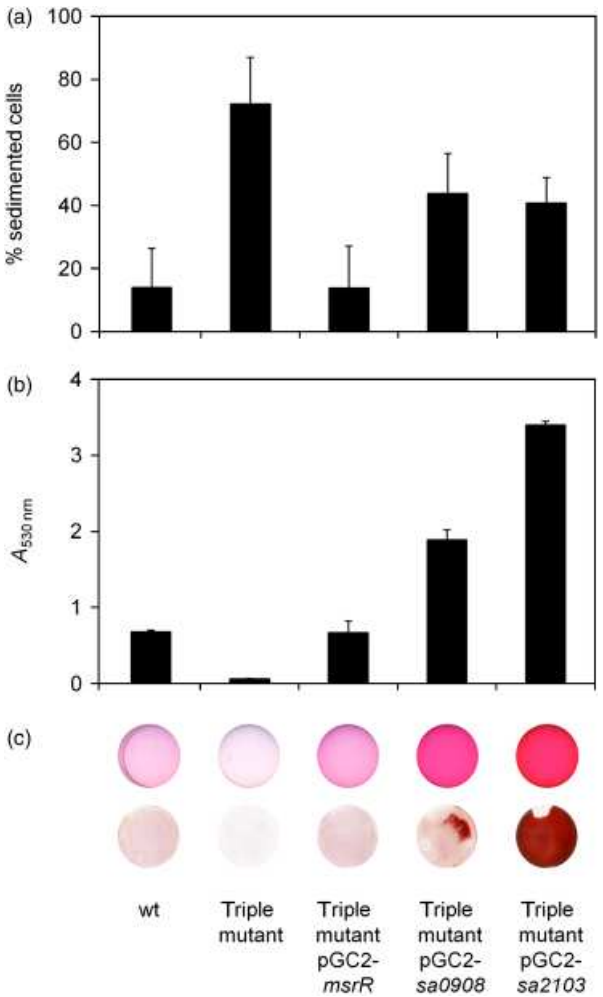

Fig. 4. Agglutination and biofilm formation. (a) Percentage of bacteria sedimented within 1 h. Mean and SD of three independent experiments. (b) Quantification of the staining expressed as the mean and SDs from three experiments. (c) Lower part, safranin stain of biofilm; upper part, dissolved stain.

Virulence of LCP mutants

To compare the contribution of the staphylococcal LCP proteins to virulence, single and double mutants were tested in a *C. elegans* killing assay. Nematode killing was most strongly attenuated in *msrR* mutants, followed by *sa0908* mutants, while *sa2103* deletion had no apparent effect on virulence. In double mutants, *sa0908* or *sa2103* deletion, combined with *msrR* deletion, reduced virulence even further (Fig. 5).

Discussion

The three *S. aureus* membrane proteins with a conserved extracellular LCP domain clearly play an important role in septum formation and cell division.

The deletion of the individual MsrR, SA0908 and SA2103 proteins had small, but distinct effects on growth and cell envelope properties; however, the triple mutant lacking all three proteins was barely viable, growth was severely impaired and temperature sensitive and cells formed large amorphous complexes containing multiple incomplete septa. Phenotypically, the triple mutant cells were similar to those of an *S. pneumoniae* LytR mutant (Johnsborg & Havarstein, 2009), supporting the hypothesis that LCP genes are essential for optimal, ordered cell division. Optimal cell growth and separation is achieved through the highly coordinated actions of cell wall synthesis and hydrolysis enzymes (Antignac *et al.*, 2007). The extremely low levels of induced autolysis in the triple mutant, indicating impaired murein hydrolase function, correspond with the TEM pictures showing irregular placement of division septa and failure of cell separation. The deletion of genes involved in autolysis is known to cause cells to become enlarged and irregularly shaped (Kajimura *et al.*, 2005). The lack of all

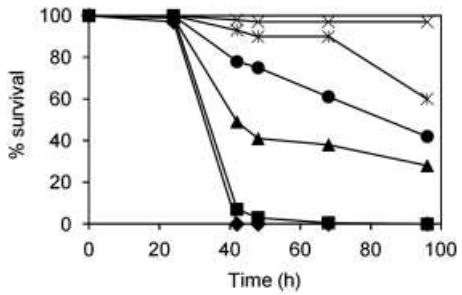

Fig. 5. Nematode-killing assay. Survival of nematodes fed *S. aureus*. Full square, MSSA1112; full circle, *msrR* mutant JH100; full triangle, *sa0908* mutant RH53; full diamond, *sa2103* mutant PS47; cross, *sa0908/msrR* mutant RH72; and star, *sa2102/msrR* mutant PS60. Ninety nematodes were observed per strain. Representative data of three independent experiments.

three LCP proteins may deplete the cells of envelope structures required to correctly localize autolytic enzymes or PBPs, functions partially attributed to wall teichoic acids (Atilano *et al.*, 2010; Schlag *et al.*, 2010). The determination of the cellular localization of the LCP proteins may shed more light on their involvement in autolysis. Further characterization of the cell physiology and envelope structure/composition of all mutants could further define the individual functions of the LCP proteins.

The severely defective growth phenotype and marked temperature sensitivity of the triple mutant could be rescued to different degrees by any one of the three proteins, with MsrR being the most efficient at restoring cell separation and decreasing temperature sensitivity. Partial restoration of growth rate and improved cell division in SA0908- and SA2103-complemented triple mutants suggested that while cells cannot grow optimally in the absence of MsrR, cell division is considerably enhanced in the presence of at least one of the three proteins. The reintroduction of any LCP protein also restored biofilm formation of the triple mutant and reduced its sedimentation rate.

Almost all phenotypes were, however, complemented to markedly different degrees by the three proteins, once again indicating that while there might be some functional overlap between these proteins, they appear to play distinct roles. Although the proteins are not completely redundant, they appear to be able to substitute for one another to varying degrees, which could ensure against complete loss of function and allow *S. aureus* greater flexibility in maintaining cell division. Functional diversification among these proteins could be linked to their sequence-based phylogenetic grouping (Hubscher *et al.*, 2008); however, currently, there are not enough data on specific members of this protein family to support this hypothesis.

In *S. pneumoniae*, the deletion of LytR was thought to only be viable because of a suppressor mutation(s) (Johnsborg & Havarstein, 2009). A similar compensatory mutation may have occurred in *S. aureus* to facilitate viability of the triple mutant, which could explain why phenotypes of triple mutants complemented with individual LCP proteins did not exactly match the genotypically equivalent double mutants; for example, the triple mutant complemented with SA0908 did not have a phenotype identical to the MsrR/SA2103 double mutant. Phenotypic differences could also be due to the altered copy number of genes expressed from multicopy plasmids.

Upregulation of these proteins, as part of the *S. aureus* cell wall stress stimulon, suggests that they either help to protect cells against cell wall damage or help to maintain cell division in the presence of cell wall-active antibiotics. A change in the phospholipid composition was shown to affect the amounts of two of the three LCP proteins in *S. aureus* (Sievers *et al.*, 2010). Many LCP homologues from other species are also upregulated under stress conditions (Mella-Herrera *et al.*, 2010; and reviewed in Hubscher *et al.*, 2008).

Decreased β-lactam resistance in several of the mutants studied here further suggests that these proteins provide some protection against cell wall-active β-lactam antibiotics. MsrR was also one of the loci found to contain point mutations after *in vitro* selection for decreased glycopeptide susceptibility by passage on imipenem and teicoplanin (Kato 2010). Although the relevance of these point mutations has not been analysed, possible alterations in MsrR either enhancing or inactivating its function could be contributing to the resulting GISA phenotypes.

This is the first time that the roles of all LCP proteins from a bacterial species with multiple LCP homologues have been investigated. The phenotypes of these three proteins and previously characterized members of this protein family suggest that LCP proteins play important, although as yet unknown, roles in cell division and cell envelope maintenance. Here, we showed that defects in cell division and other changes in cell envelope characteristics generally increased, while virulence decreased, when two or more of the LCP proteins were mutated. Finally, the depletion of all LCP proteins appeared to be extremely detrimental to the cell if not potentially lethal.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers.

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